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A novel rat model of gestational diabetes induced by intrauterine programming is associated with alterations in placental signaling and fetal overgrowth

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ABSTRACT

A family history of diabetes predisposes to gestational diabetes mellitus (GDM). We hypothesized that female offspring of rats with pre-gestational diabetes will develop GDM, a pathology associated with fetal overgrowth and altered placental signaling. We found normal glycemia and insulinemia in the offspring from pre-gestational diabetic rats at three months of age. However, consistent with GDM, maternal hyperglycemia and hyperinsulinemia and increased fetal weight were evident when compared to controls. In this intrauterine programmed GDM model, the placentas showed alterations in mTOR pathway: unchanged phosphorylation of 4EBP-1 and PKCα despite reduced total expression of 4EBP-1 and PKCα, and increased phosphorylation of SGK1. GDM placentas also showed reduced expression of PPARα and PPARγ, and increased lipoperoxidation, nitric oxide production and peroxynitrite-induced damage. We conclude that exposure of maternal diabetes *in utero* programs GDM in the female offspring, leading to a GDM model associated with impaired placental signaling pathways, increased pro-oxidant/pro-inflammatory environment and fetal overgrowth.

1. Introduction

Gestational diabetes mellitus (GDM) is a common pregnancy disease affecting more than 8% of the pregnant women in many populations, and is associated with short and long-term adverse consequences for the mother and the fetus (Ashwal and Hod, 2015; Pu et al., 2015; Wang et al., 2013). GDM affects the fetal growth pattern, and macrosomia has been observed in 15–45% newborns of GDM mothers (Kc et al., 2015). Alterations in nutrient transfer and oxidative/inflammatory pathways characterize GDM placentas, and have been related to the adverse intrauterine programming (Diaz et al., 2014; Lappas et al., 2011). Indeed, GDM increases the risk of metabolic syndrome and type 2 diabetes in the offspring (Bellamy et al., 2009).

To explore the mechanisms linking GDM to adverse outcomes and study novel intervention strategies animal models relevant to the clinical condition are required. Specifically, a clinically relevant model of GDM should replicate common characteristics of the human condition, including hyperglycemia detected for first time in pregnancy as a result of insulin resistance in combination with some degree of beta cell insufficiency and increased fetal weight. Unfortunately, few such models are currently available (Jawerbaum and White, 2010).

It is well-known that a family history of diabetes is a risk factor for developing GDM (Buchanan and Xiang, 2005). In addition, there is evidence that experimental models of pre-gestational diabetes, obesity and intrauterine growth restriction induce metabolic

Abbreviations: 4EBP1, eukaryotic initiation factor 4E-binding protein 1; GDM, gestational diabetes mellitus; mTOR, mammalian target of rapamycin; mTORC1, mTOR Complex 1; mTORC2, mTOR Complex 2; S6K1, S6 kinase 1; NO, nitric oxide; PKCα, protein kinase Cα; PPARα, peroxisome proliferator activated receptor α; PPARγ, peroxisome proliferator activated receptor γ; PPARδ, peroxisome proliferator activated receptor δ; rpS6, ribosomal protein S6; SGK1, serum- and glucocorticoid-inducible kinase 1; TBARS, thiobarbituric acid reactive substances.

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alterations in the offspring leading to type 2 diabetes (Capobianco et al., 2015; Zambrano and Nathanielsz, 2013). There is a strong association between type 2 diabetes and GDM in the genetic background, and, in several populations, more than 30% of women that had GDM will develop type 2 diabetes within 5 years of delivery (Bellamy et al., 2009; Huopio et al., 2013; Kim et al., 2002).

Fetal overgrowth is a frequent complication of GDM related to the alterations in growth factors and sustained by an increase in placental nutrient transport (Hiden et al., 2009; Jansson et al., 2002; Lappas et al., 2011; Magnusson et al., 2004). Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase which has been proposed to function as a placenta nutrient sensor (Jansson et al., 2012; Roos et al., 2009). mTOR is the catalytic subunit of two complexes named mTOR Complex 1 (mTORC1) and 2 (mTORC2), and its signaling leads to phosphorylation cascades resulting in an increase in amino acid transport, protein synthesis and cellular proliferation (Bracho-Valdes et al., 2011). Downstream target proteins phosphorylated by mTORC1 and related to increased protein synthesis are p70 S6 kinase 1 (S6K1), the ribosomal protein S6 (rpS6) and the eukaryotic initiation factor 4E-binding protein 1 (4EBP1) (Laplante and Sabatini, 2013). Activation of placental mTORC1 has been observed in association to maternal obesity and GDM and is related to fetal overgrowth (Jansson et al., 2013; Perez-Perez et al., 2013).

mTORC2 signaling has been less studied in the placenta. mTORC2 direct targets include protein kinase Cα (PKCα) and serum- and glucocorticoid-inducible kinase 1 (SGK1) (Heikamp et al., 2014; Oh and Jacinto, 2011). mTORC2 activation is involved in the regulation of cell volume, growth, differentiation and motility in different cell types (Betz and Hall, 2013; Oh and Jacinto, 2011). Furthermore, recent works has demonstrated that mTORC2 signaling is a positive regulator of placental amino acids transport (Rosario et al., 2013).

A common feature of obesity, pre-gestational diabetes, GDM and intrauterine growth restriction is the generation of a pro-oxidative/pro-inflammatory environment, which may provide a mechanism underpinning intrauterine programming of metabolic diseases (Higa and Jawerbaum, 2013; Lappas et al., 2011; Pantham et al., 2015). GDM is characterized by elevated levels of maternal and placental pro-inflammatory cytokines, and increased markers of oxidative stress, nitrative stress and infiltration of immune cells in the placenta (Lappas et al., 2011; Mrizak et al., 2014). In experimental models of diabetes and in women with GDM and type 2 diabetes there are reduced placental expression of peroxisome proliferator activated receptor α (PPARα) and PPARγ (Capobianco et al., 2013; Holdsworth-Carson et al., 2010; Jawerbaum et al., 2004). PPARs are ligand activated transcription factors that transactivate and transrepress multiple genes involved in the regulation of diverse metabolic, developmental and anti-inflammatory pathways (Wahli and Michalik, 2012). Previous experimental work has addressed the relevance of impaired PPARs in diabetic pregnancies and the role of their activation in the prevention of the pro-oxidant and pro-inflammatory intrauterine environment (Jawerbaum and Capobianco, 2011; Kurtz et al., 2014; Martinez et al., 2012).

In this work, we hypothesized that 1) adverse intrauterine programming in an experimental model of mild pre-gestational diabetes (F0) leads to GDM in the female offspring (F1) and, 2) experimental GDM pregnancies are characterized by fetal overweight and placental alterations in regulators of nutrients transfer and a pro-oxidative/pro-inflammatory environment. Thus, we studied the female offspring of control rats compared with the female offspring of rats with pre-gestational diabetes before mating and at term pregnancy. We analyzed metabolic and growth parameters, as well as placental mTOR signaling, lipoperoxidation, nitric oxide production, peroxynitrite-induced damage and PPARs.

2. Methods

2.1. Animals

Albino Wistar rats bred in our animal facility were fed *ad libitum* with commercial rat chow (Asociación Cooperativa Argentina, Buenos Aires, Argentina). As shown in Fig. 1, to induce diabetes in the F0 generation, pups were injected with streptozotocin (90 mg/kg, s.c, Sigma–Aldrich, St. Louis, MO, USA) diluted in citrate buffer (0.05 M, pH 4.5, Sigma–Aldrich) at postnatal day 2, as previously described (Jawerbaum and White, 2010; Kurtz et al., 2014). Control animals were injected with citrate buffer alone. The presence of pre-gestational diabetes, defined as fasting blood-glucose higher than 130 mg/dl, was confirmed in two-month-old rats prior to mating. The characteristics of this mild pre-gestational diabetic model have been reported previously (Jawerbaum and White, 2010; White et al., 2015).

Control and diabetic female F0 rats were mated with control males. The first day of pregnancy was confirmed by the presence of sperm cells in vaginal smears. The animals were allowed to deliver and the offspring (F1) were kept with their mothers until weaning. No treatments were performed in the F1 animals, which were studied at three to four months of age. One group of female animals was studied in the non-pregnant state and the other mated with control males and studied at day 21 of pregnancy (Fig. 1). GDM animals were defined by fasting blood-glucose lower than 130 mg/dl before pregnancy and higher than 130 mg/dl in pregnancy, defined considering the media and standard deviation of fasting glycemia values in 21-day pregnant control rats. The animal protocol was approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAL, Resolution CD N° 1497/2013), School of Medicine, University of Buenos Aires, and conducted according to the Guide for the Care and Use of Laboratory Animals, US National Institutes of Health (NIH Publication, 8th Edition, 2011) http://www.ncbi.nlm.nih.gov/books/NBK54050/?report=reader.

All animals were euthanized by decapitation. The fetuses and placentas were weighed. Plasma from non-pregnant rats, pregnant rats on day 21 of pregnancy and 21-day fetuses was obtained and was stored at −80 °C. Two whole placentas per rat, selected at random, were fixed in formaldehyde and subsequently embedded in paraffin for immunohistochemical analysis of protein nitrotyrosylation. Two additional whole placentas per rat were snap frozen and stored at −80 °C for further analysis of nitric oxide production and lipoperoxidation. Another two whole placentas per rat were sliced and preserved in RNAlater for gene expression studies. The remaining whole placentas were pooled and homogenized in ice-cold buffer D (250 mM sucrose, 10 mM Hepes-Tris, pH 7.4 with protease and phosphatase inhibitors) and stored at −80 °C until use for Western blot analysis of proteins.

2.2. Metabolic assays

In the F0 generation, blood-glucose was measured by the Accu-Chek reagent strips and a glucometer Accu-Chek (Bayer Diagnostics, Buenos Aires, Argentina) in samples obtained from the tail vein of the adult rats. In the F1 generation, maternal and fetal plasma concentrations of glucose, triglycerides and cholesterol were measured in plasma by spectrophotometric enzymatic assays (Wiener lab. Rosario, Argentina) (n = 10 rats in each experimental group) (White et al., 2015). Plasma insulin was determined using a commercial assay kit (Mercodia Ultrasensitive Rat Insulin ELISA kit, Uppsala, Sweden), according to the manufacturer's instructions, as previously described (White et al., 2015).

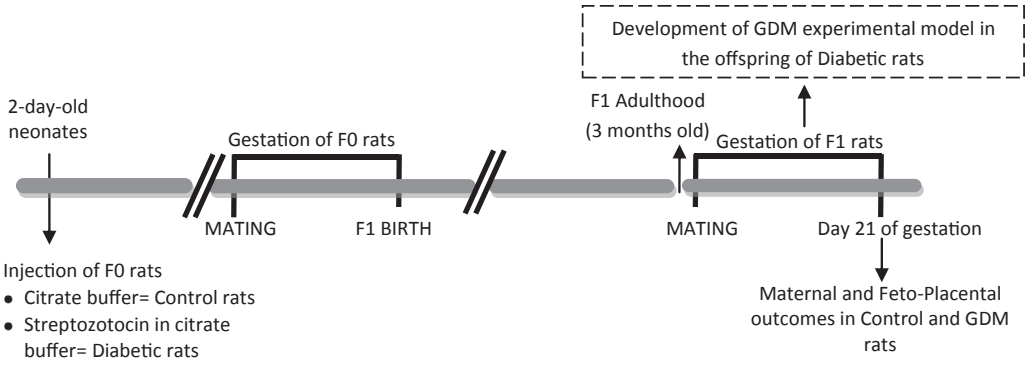


Fig. 1. Experimental Design: Pre-gestational diabetes in the F0 was induced in Wistar rats by neonatal administration of streptozotocin. Control and diabetic female rats were mated with control males. At three months of age and after metabolic evaluation, the female offspring from control and diabetic rats were mated and studied on day 21 of pregnancy.

2.3. Western blot analysis

Proteins from placental homogenates (pooled whole placentas from n = 7 rats in each experimental group) were separated by SDS-PAGE electrophoresis and transferred to PVDF membranes (35V constant, overnight at 4 °C), as described previously (Gaccioli et al., 2013). The membranes were then stained with the Amido Black staining solution for total proteins (Sigma–Aldrich) to confirm equal loading and transfer. Blocking was carried out for 1 h at room temperature in 5% non-fat milk in TBS-tween and membranes were incubated in primary antibody (diluted in 1% BSA in TBS-Tween) overnight at 4 °C. The expression of the following proteins in placental homogenate was determined using Cell Signalling Technology antibodies: total and phosphorylated S6K1, total and phosphorylated rpS6, total and phosphorylated 4EBP-1, total and phosphorylated PKCα, and total and phosphorylated SGK1. Besides, nitrated proteins were determined using Millipore anti-nitrotyrosine antibody. After washing, the membranes were incubated with the appropriate peroxidase conjugated secondary antibody and visualized using ECL detection solution (Thermo Scientific) and a G:BOX gel imaging system (Syngene). Densitometry analysis was performed with ImageJ software. The expression of the target protein in each individual lane was normalized for the total protein staining to adjust for unequal loading. The mean of all the samples was calculated and the expression of the target protein in each sample (target/total protein density) was then calculated as percentage of that mean.

2.4. Lipoperoxidation measurement

Lipoperoxidation was assessed by evaluating the concentrations of thiobarbituric acid reactive substances (TBARS), a method widely used to assess peroxidation of fatty acids, in whole placentas from control and GDM rats (two placentas for each rat, n = 8 rats in each experimental group). Briefly, each placenta was homogenized in 100 mM Tris–HCl buffer (0.1 mM, pH: 7.4) and TBARS evaluated as previously described (Kurtz et al., 2014).

2.5. Nitric oxide production

Nitric oxide production was determined by measuring the concentration of its stable metabolites nitrates/nitrites, as reported previously (Kurtz et al., 2014), in whole placentas from control and GDM rats (two placentas for each rat, n = 8 rats in each experimental group). Briefly, each placenta was homogenized in 1 ml Tris–HCl buffer pH 7.6, and an aliquot was separated for protein analysis. After reducing nitrates to nitrites using nitrate reductase enzyme, nitrites were measured by using a commercial assay kit (Cayman Chemical Co. Ann Arbor, MI, USA), according to the manufacturer's instructions.

2.6. Nitrotyrosine and PPARs immunostaining

Immunostaining of nitrotyrosine (an index of peroxynitrite-induced damage (Lappas et al., 2011)) and of PPARs was evaluated in the placentas from control and GDM rats (n = 6 in each experimental group). Two whole placentas for each rat were paraffinized and serially sliced. Then, 5 μm-thick sections were deparaffinized, rehydrated through a graded series of ethanol and the endogenous peroxidase activity was blocked. Then, the sections were processed using the anti-nitrotyrosine mouse monoclonal antibody (Millipore, Darmstadt, Germany) (1:4000 dilution) and the following anti-PPARs antibodies as primary antibodies: rabbit polyclonal antibody anti-PPARα (Cayman Chemical Company) (1:200 dilution), goat polyclonal antibody anti-PPARδ (Santa Cruz Biotechnology) (1:300 dilution) and rabbit polyclonal antibody anti-PPARγ (Santa Cruz Biotechnology) (1:50 dilution). Then, the Avidin–Biotin Complex technique (ABC) was followed and developed with 3,3'-diaminobenzidine, as reported previously (Martinez et al., 2012). Control sections were performed by omitting the primary antibody. Three entire sections per rat were examined using light microscopy by two skilled blinded observers. Immunoreactivity intensity was quantified using the ImageProPlus software. Data are shown as relative to a value of 1, arbitrarily assigned to the control. Similar results were obtained using a semiquantitative score (data not shown).

2.7. PPARs gene expression

RNA from whole placentas (two placentas per rat, n = 7 rats in each experimental group) was extracted with TriReagent (Molecular Research Center, Inc, USA) for further determination of the mRNA expression of the three PPAR isotypes by semiquantitative end-point RT-PCR, as described previously (Kurtz et al., 2014). RNA concentration and purity were assessed in a NanoDrop equipment (NanoDrop 1000 Spectrophotometer, Thermo Scientific, USA). cDNA was synthesized incubating 2 μg of extracted RNA in a first-strand buffer containing MMLV enzyme from Promega (Buenos Aires, Argentina), random primer hexamers (Promega) and each of all four dNTPs (Invitrogen, CA, USA) in accordance with the MMLV

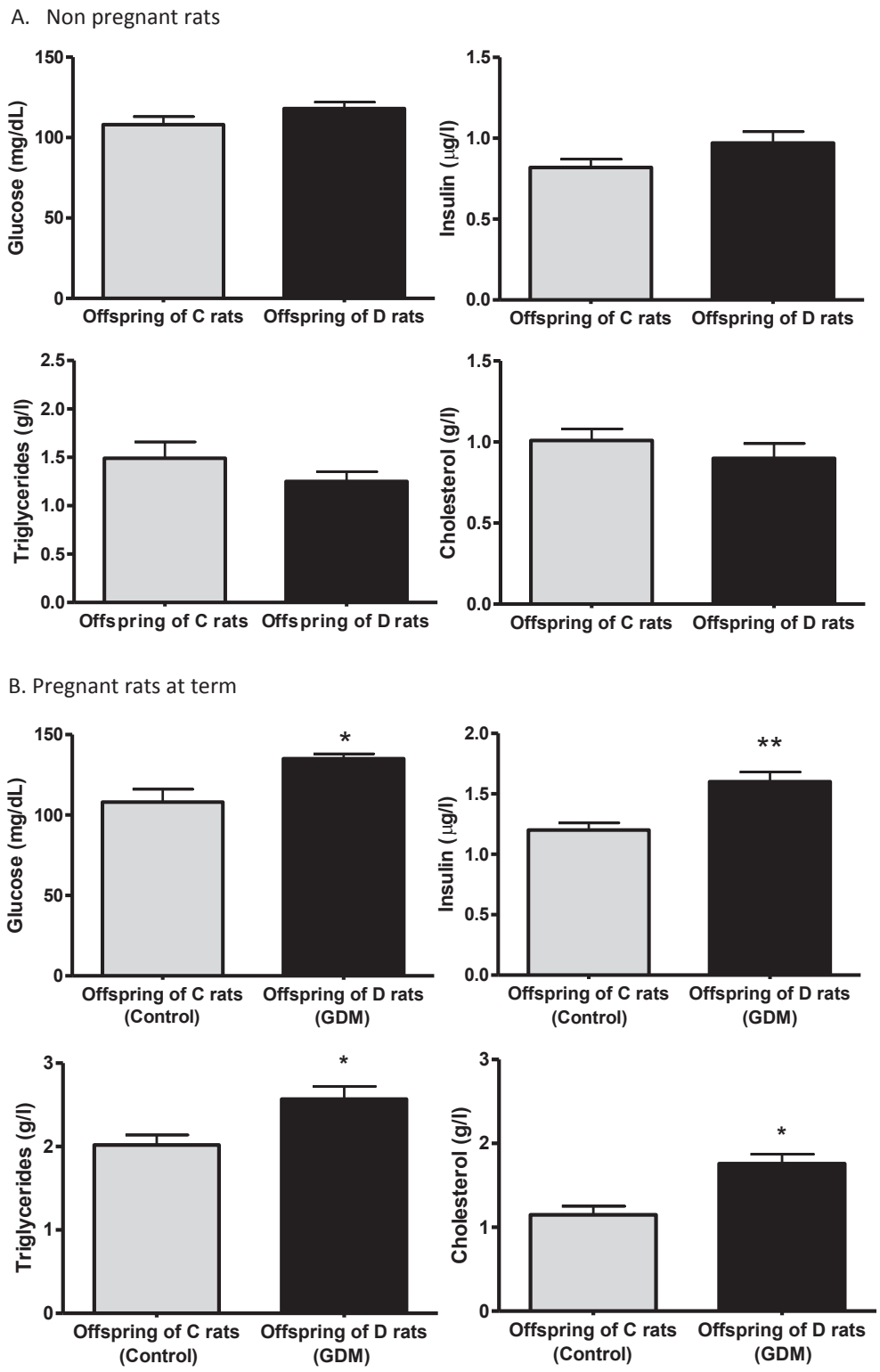


Fig. 2. Metabolic parameters in the non-pregnant and pregnant rats. **A**. Plasma glucose, insulin, triglycerides and cholesterol in the non-pregnant offspring of control (C) and pre-gestational diabetic (D) rats at three months of age. **B**. Plasma glucose, insulin, triglycerides and cholesterol in the offspring of control (C) and pre-gestational diabetic (D) rats on day 21 of pregnancy (3–4 months old). The results are consistent with GDM in the pregnant offspring of diabetic rats. Values represent mean ± SEM obtained from 10 rats in each experimental group. Statistical analysis: Student *t* test: $^{*}p < 0.05$, $^{**}p < 0.01$ vs control.

manufacturer's instructions. Controls performed in the absence of MMLV or sample were run in parallel to assure no DNA contamination. The reaction mixture was incubated at 37 °C for 60 min and then at 70 °C for 15 min cDNA (2 μl, selected to work within the linear range) was amplified by PCR in a buffer containing dNTPs, magnesium chloride solution, Taq polymerase (GoTaq Polymerase, Promega) and each specific primer in accordance with the Taq polymerase manufacturer's instructions. The following primers

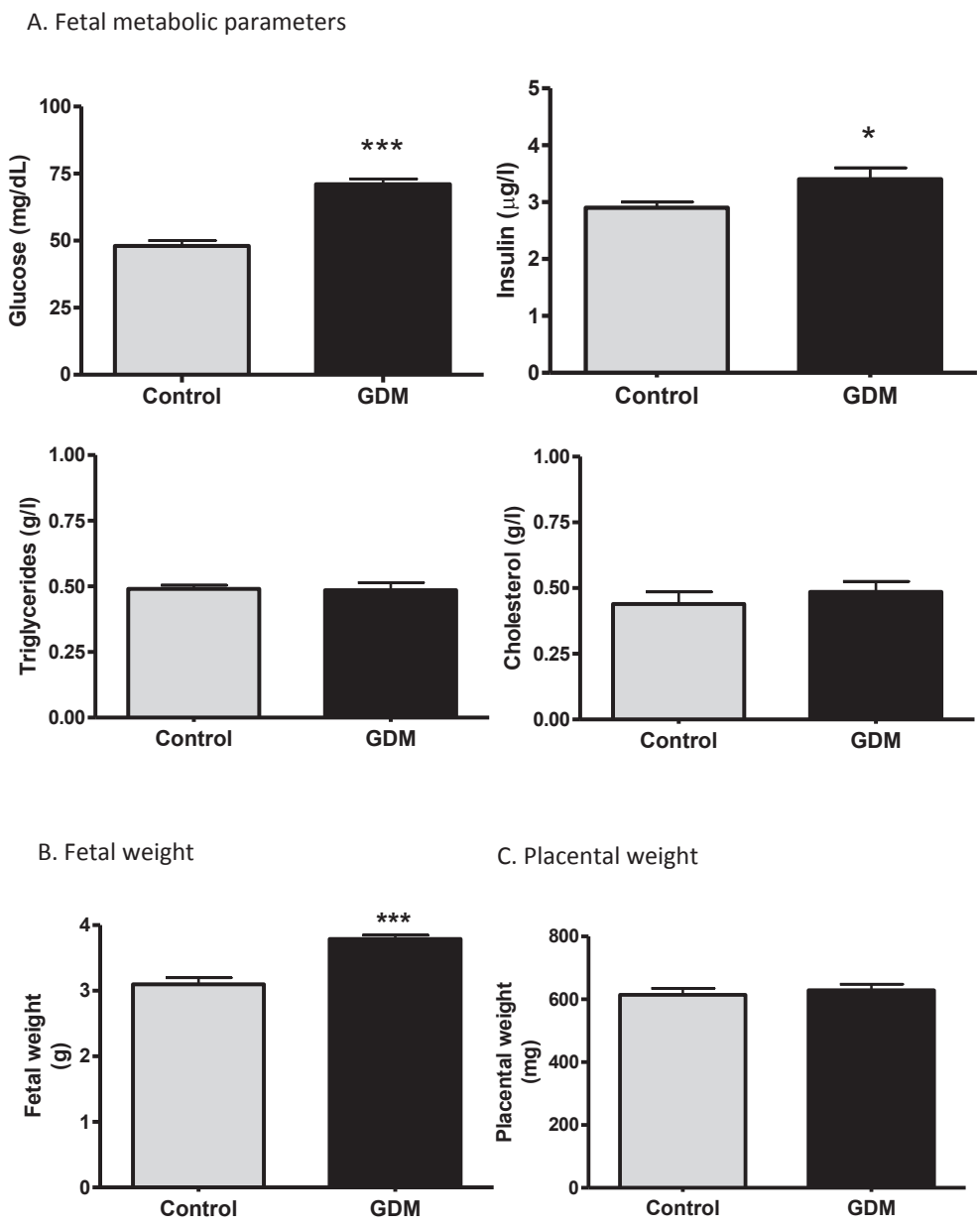


Fig. 3. Metabolic parameters and weight for the fetuses from control and GDM rats. **A**. Plasma glucose, insulin, triglycerides and cholesterol in 21-day-old fetuses from control and GDM rats. Fetal weight (**B**) and placental weight (**C**) in control and GDM rats on day 21 of pregnancy. Values represent mean ± SEM obtained from 10 rats in each experimental group. Statistical analysis: Student *t* test: $^{*}p < 0.05$, $^{***}p < 0.001$ vs control.

were used for PPARα: forward: 5′-TCACACAATGCAATCCGTTT-3′ and reverse: 5′-GGCCTTGACCTTGTTCATGT-3′); PPARδ: forward: 5′-GAGGGGTGCAAGGGCTTCTT-3′ and reverse: 5′-CACTTGTTGCGGTTCTTCTTCTG-3′; PPARγ: forward: 5′-CAGATC-CAGTGGTTGCAG-3′ and reverse 5′-GTCAGCGGACTCTGGATT-3′ and ribosomal protein L30: forward: 5′-CCATCTTGGCGTCTGATCTT-3′ and reverse: 5′-GGCGAGGATAACCAATTTC-3′, which was used as an internal control. All primers were designed using the Primer 3 software and used previously (Kurtz et al., 2014). Each PCR cycle consisted of denaturation at 95 °C for 15 s, primer annealing at 58 °C for 30 s and extension at 72 °C for 15 s. The numbers of cycles were 36 for PPARα, 32 for PPARδ, 38 for PPARγ and 26 for L30. The resulting products were separated on a 2% agarose gel and stained with SYBR Safe (Invitrogen). The density of the bands was quantified by image analysis with Image J software and relativized to L30 values (housekeeping gene used as an internal control). Ribosomal protein S18 was also used as an internal control and the results were similar to those observed with L30 (data not shown).

2.8. Statistical analysis

Data are presented as the mean ± standard error (SEM). Groups were compared by Student *t* test. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. Metabolic characterization of an experimental model of GDM

We have previously shown that from the fifth month of age the

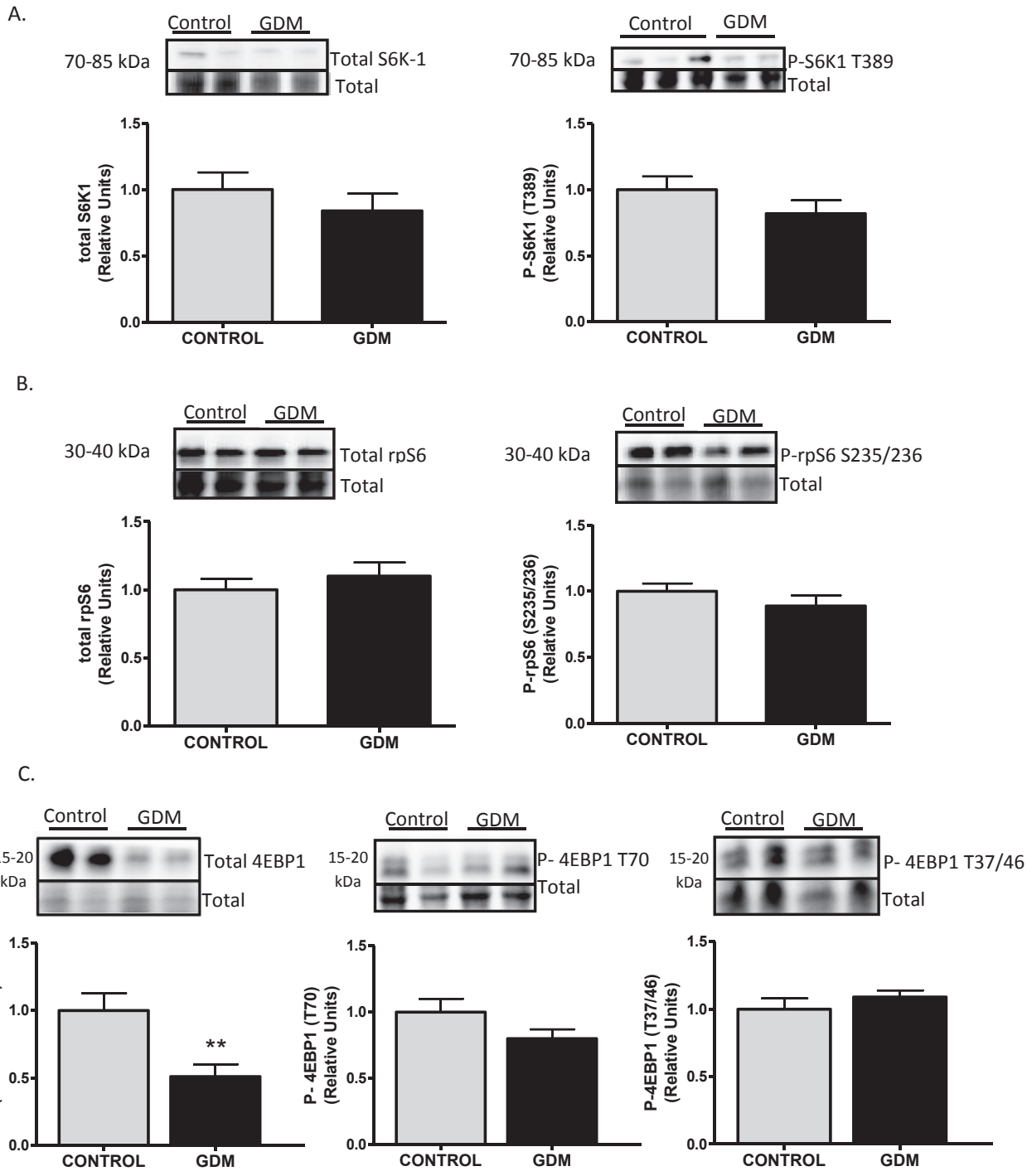


Fig. 4. Expression of proteins involved in mTORC1 pathway in the placenta from control and GDM rats. **A**. Representative Western blots and summary of phosphorylated and total S6K1. **B**. Representative Western blots and summary of phosphorylated and total rpS6. **C**. Representative Western blots and summary of phosphorylated and total 4EBP-1. Values represent mean ± SEM obtained from 7 rats in each experimental group. Statistical analysis: Student *t* test: $^{**}p < 0.01$ vs control.

offspring of mild pre-gestational diabetic rats are hyperglycemic and have elevated fasting plasma insulin levels (Capobianco et al., 2015). In this work we found that at three months of age plasma concentrations of glucose, insulin, triglycerides and cholesterol in the offspring from mild pre-gestational diabetic rats were not different when compared to the offspring of control rats (Fig. 2A). In contrast, at term pregnancy, the offspring from mild diabetic rats showed increased fasting plasma glucose, insulin, triglycerides and cholesterol when compared to controls ($p < 0.05$, Fig. 2B). Thus, using the definition of fasting blood-glucose lower than 130 mg/dl before pregnancy and higher than 130 mg/dl in pregnancy, these F1 animals had GDM. In addition, the fetuses from these gestational diabetic rats were hyperglycemic ($p < 0.001$) and hyperinsulinemic ($p < 0.05$), although no changes in fetal triglyceride and cholesterol plasma levels were observed when compared to controls (Fig. 3A). Fetal weight was increased in GDM rats when compared to controls

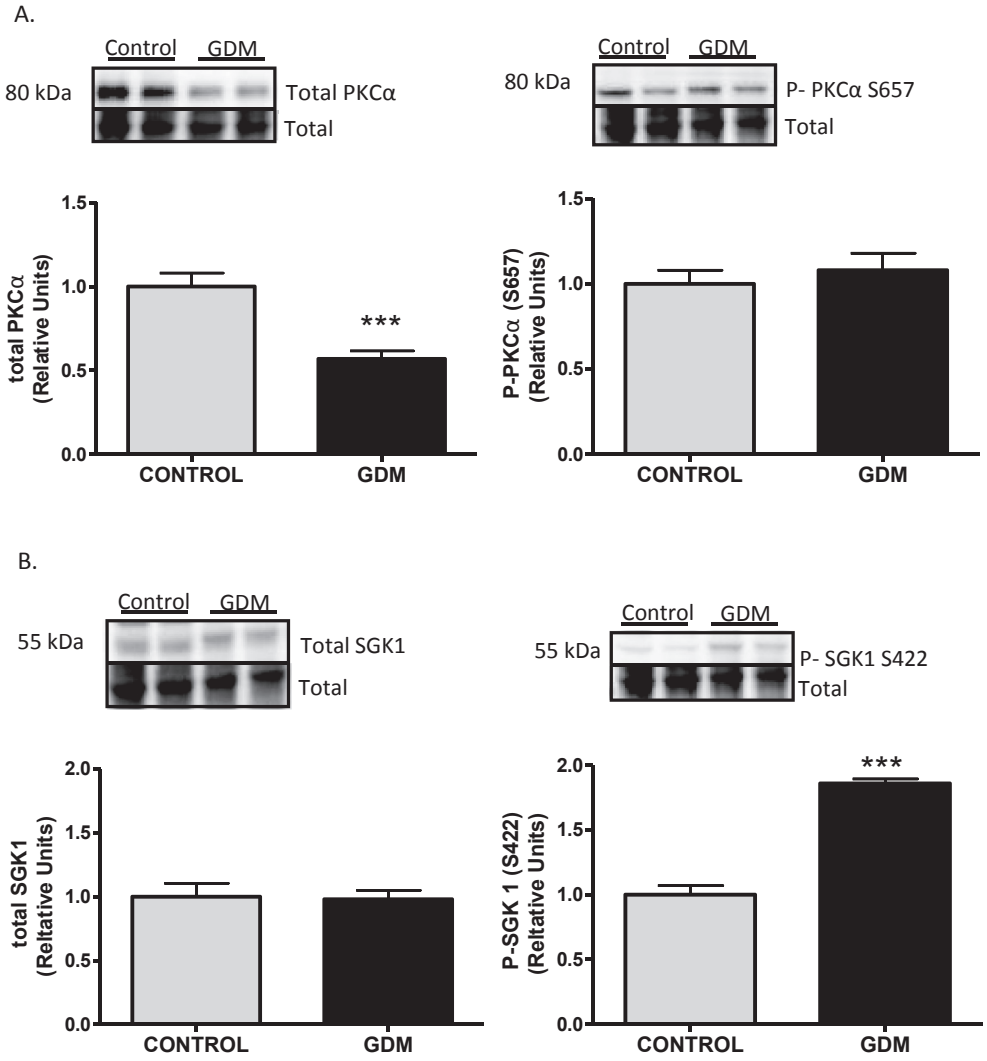


Fig. 5. Expression of proteins involved in mTORC2 pathway in the placenta from control and GDM rats. **A**. Representative Western blots and summary of phosphorylated and total PKCα. **B**. Representative Western blots and summary of phosphorylated and total SGK1. Values represent mean ± SEM obtained from 7 rats in each experimental group. Statistical analysis: Student *t* test: ***$p < 0.001$ vs control.

($p < 0.05$), however placental weights were unaltered (Fig. 3B and C).

3.2. mTOR pathway signaling

To investigate pathways that have been related to increased fetal weight in human gestational diabetes (Jansson et al., 2013), we focused on determining the activity of placental mTOR pathway in the offspring of control and diabetic pregnant rats (F1 generation). We found no change in phosphorylation or total expression of S6K1 and rpS6 in the placenta from GDM rats compared to controls (Fig. 4 A and B). In contrast, although 4EBP-1 phosphorylation was not affected by GDM, we observed reduced total expression of 4EBP-1 in the placenta from GDM rats ($p < 0.01$, Fig. 4C).

Next we determined the effect of GDM on placental mTORC2 signaling. PKCα phosphorylation was unchanged but total expression of PKCα was markedly decreased ($p < 0.001$). In addition, phosphorylation of SGK1 was increased whereas total SGK1 expression was unaffected in the placenta from GDM rats ($p < 0.001$, Fig. 5). Collectively, this data is consistent with an activation of the placental mTOR pathway.

3.3. Markers of oxidative and nitrative stress in the placenta

Because GDM in women is characterized by an intrauterine pro-oxidative/pro-inflammatory intrauterine environment (Lappas et al., 2011), we tested the hypothesis that experimental GDM induced by intrauterine programming is associated with increased placental oxidative and nitrative stress. In the F1 generation, we found that the concentrations of TBARS, a widely used marker of lipoperoxidation and oxidative stress (Negre-Salvayre et al., 2010), was increased in the placenta from GDM rats compared to controls ($p < 0.01$, Fig. 6A). Nitric oxide production, assessed by determining the concentration of nitrates-nitrites, stable metabolites of nitric oxide, was increased in the placenta from GDM rats compared to controls ($p < 0.05$, Fig. 6B). Moreover, protein nitrotyrosylation, produced by the potent oxidant peroxynitrite, formed in the presence of excessive nitric oxide and reactive oxygen species, was found to be increased in GDM placentas ($p < 0.01$, Fig. 6C and $p < 0.05$, 6D).

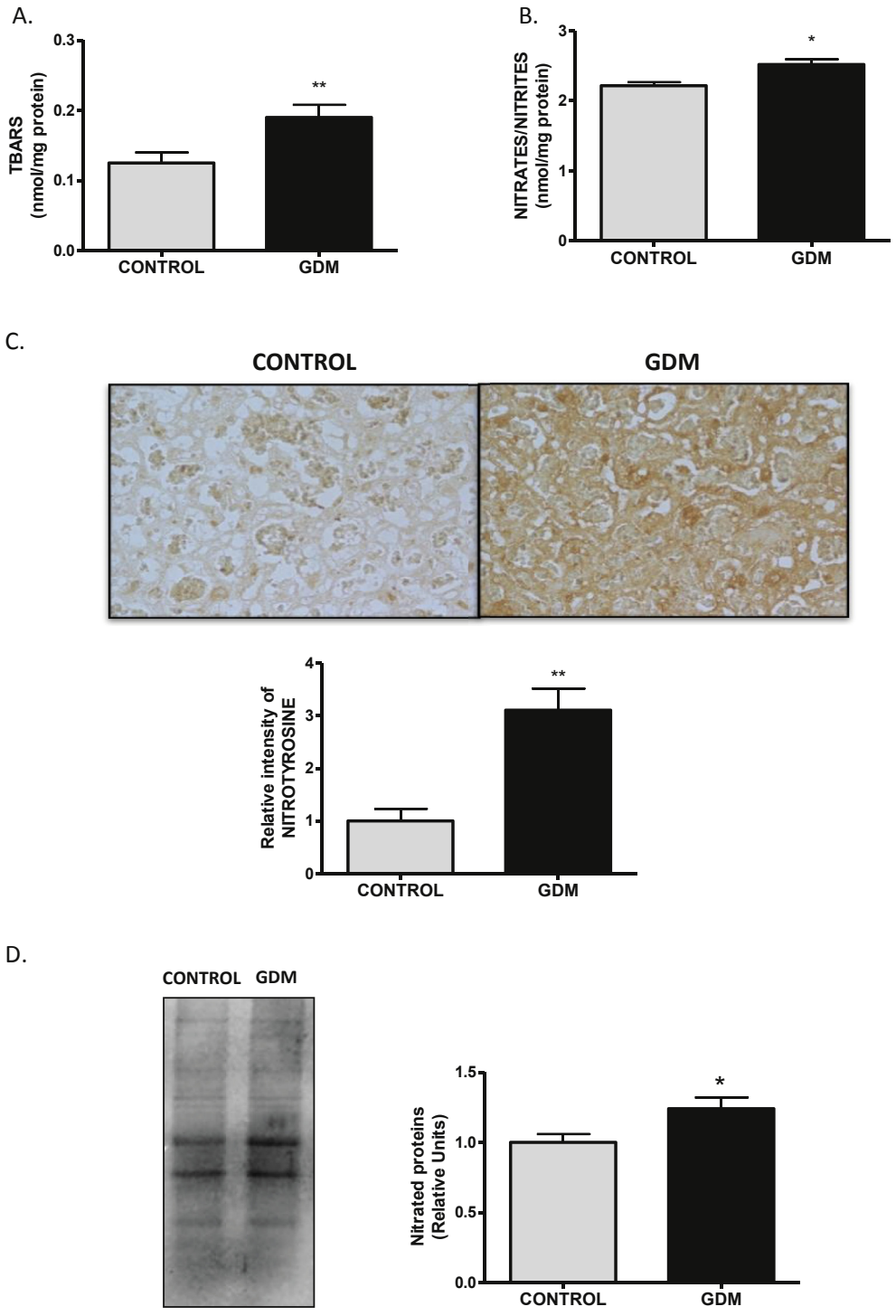


Fig. 6. Lipoperoxidation, nitric oxide production and peroxynitrite-induced damage in the placenta from control and GDM rats. **A**. Lipoperoxidation, measured as TBARS concentrations. **B**. Nitric oxide production, measured as the concentrations of NO stable metabolites nitrates and nitrites. **C**. Peroxynitrite-induced damage, evaluated through nitrotyrosine immunolocalization. Representative microphotographs and densitometry analysis are shown. **D**. Peroxynitrite-induced damage, evaluated through protein nitration by Western blot. Representative Western blot and densitometry analysis are shown. Values represent mean ± SEM obtained from 6 to 8 rats in each experimental group. Statistical analysis: Student *t* test: $^{*}p < 0.05$, $^{**}p < 0.01$ vs control.

3.4. Placental PPARs gene expression

To investigate putative pathways involved in the regulation of increased pro-oxidative/pro-inflammatory markers in the placenta from GDM rats, we determined the gene expression of placental PPARs (Jawerbaum and Capobianco, 2011). We found reduced expression of PPARα ($p < 0.05$) and PPARγ ($p < 0.05$) in the placentas from GDM rats as compared to controls, whereas placental PPARδ was unaffected by GDM (Fig. 7). Reduced protein expression of PPARα ($p < 0.01$) and PPARγ ($p < 0.001$) and no changes in PPARδ

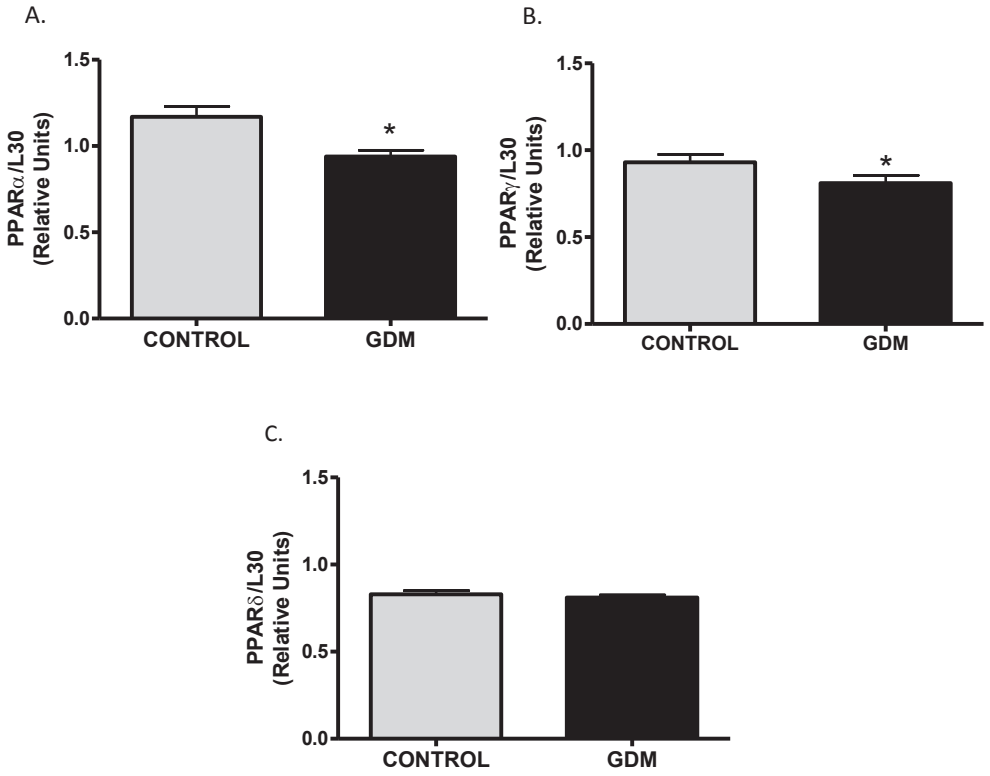


Fig. 7. Expression of PPARs in the placenta from control and GDM rats. **A**. PPARα expression. **B**. PPARγ expression. **C**. PPARδ expression. Values represent mean ± SEM obtained from 8 rats in each experimental group. Values represent mean ± SEM obtained from 7 rats in each experimental group. Statistical analysis: Student *t* test: $^{*}p < 0.05$.

protein expression were observed in the placentas from rats that developed GDM (F1 generation) as a result of intrauterine programming when compared to controls (Fig. 8).

4. Discussion

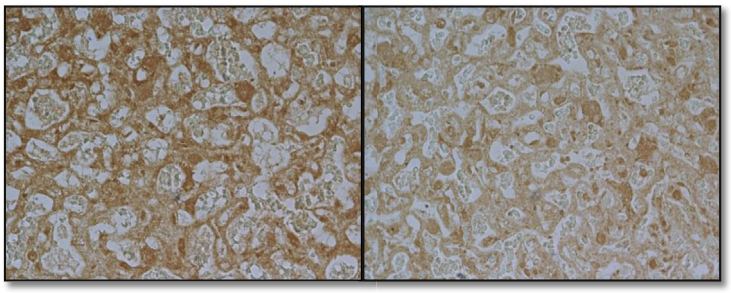
Gestational diabetes is a common pregnancy pathology, however, the mechanistic understanding of how GDM leads to adverse maternal and fetal outcomes is limited due to the paucity of clinically relevant animal models of GDM (Jawerbaum and White, 2010; Lappas et al., 2011). In this work, we characterized a novel GDM model induced by intrauterine programming leading to maternal and fetal metabolic impairments in the female offspring when pregnant. The changes are consistent with GDM and clinically relevant due to the hyperglycemia and hyperinsulinemia appearing first in pregnancy, increased fetal growth, altered placental mTOR and PPAR pathways as well as increased markers of placental oxidative and nitrative stress.

It is long recognized that GDM is associated with a marked increased risk of perinatal mortality and morbidity (Ashwal and Hod, 2015; Pu et al., 2015; Wang et al., 2013). Intrauterine programming of obesity and type-2 diabetes is well established in a multitude of epidemiological and animal experimental studies (Berends and Ozanne, 2012; Higa and Jawerbaum, 2013; Lappas et al., 2011; Zambrano and Nathanielsz, 2013). Epidemiological studies also link a family history of diabetes with increased risk of GDM (Buchanan and Xiang, 2005; Pu et al., 2015). Our finding that the offspring from mild pre-gestational diabetic rats were normoglycemic before mating at three months of age, but developed hyperglycemia and hyperinsulinemia during pregnancy suggests this is a highly relevant model for studying GDM. Furthermore, we demonstrated intrauterine programming of GDM in a mild pre-gestational diabetic model, allowing for future studies to define mechanistic links between maternal pre-gestational diabetes and GDM.

Hyperglycemia in pregnancy leads to fetal hyperglycemia and hyperinsulinemia and is associated with fetal growth (Hiden et al., 2009). We found fetal hyperglycemia and hyperinsulinemia in the GDM rats, suggesting glucose and insulin are important for fetal overgrowth in this model of GDM. Interesting, fetal circulating triglyceride and cholesterol concentrations were not changed in GDM rats despite the increased triglycerides and cholesterol levels in the maternal circulation, suggesting possible increases in fat deposition in fetal organs, an issue that will require future studies. Indeed, increased placental lipid transfer and fetal fat deposition have been found in pre-gestational experimental models of diabetes (Herrera and Ortega-Senovilla, 2014; Higa and Jawerbaum, 2013).

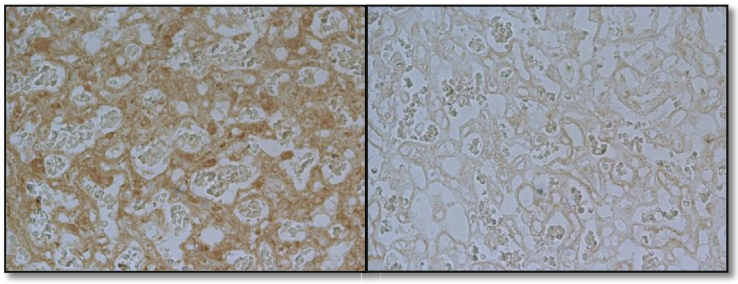
mTOR signaling is a powerful regulator of placental amino acid transfer, a key determinant of fetal growth. Accordingly, placental mTOR signaling is activated in human obesity and GDM, and inhibited in both human placentas and in experimental models of intrauterine growth restriction (Jansson et al., 2013; Perez-Perez et al., 2013; Rosario et al., 2011). In the GDM experimental model, we found evidence of activation of placental mTORC1 and 2 signaling pathways. Although GDM did not affect the activity in the S6K1/rpS6 branch of the mTORC1 pathway, total expression of 4EBP-1 was markedly reduced, without changes in 4EBP-1 phosphorylation. These changes indicate an activation of this branch of the mTORC1 pathway because 4EBP-1 is a binding protein and decreased total expression of the protein results in the release of eIF4E, leading to an activation of protein synthesis (Jansson et al., 2012; Laplante and Sabatini, 2013). Similarly, the decreased total expression of PKCα and the increased phosphorylation of SGK1 are

A.



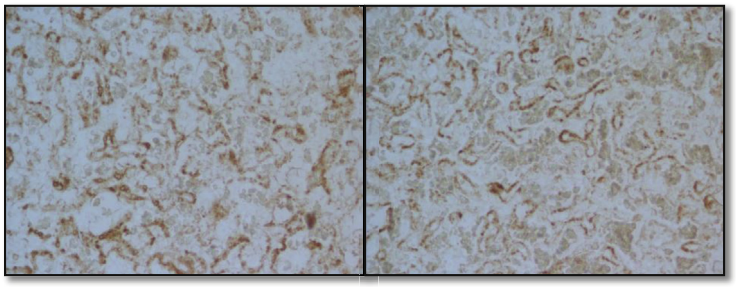
Densitometry analysis of PPARα immunostaining	
CONTROL	1 ± 0.1
GDM	0.61 ± 0.05 **

B.



Densitometry analysis of PPARγ immunostaining	
CONTROL	1 ± 0.05
GDM	0.38 ± 0.02 ***

C.



Densitometry analysis of PPARδ immunostaining	
CONTROL	1 ± 0.18
GDM	1.06 ± 0.16

Fig. 8. Protein expression of PPARs in the placenta from control and GDM rats. **A**. PPARα protein expression. **B**. PPARγ protein expression. **C**. PPARδ protein expression. Representative microphotographs and densitometry analysis are shown. Values represent mean ± SEM obtained from 6 rats in each experimental group. Statistical analysis: Student *t* test: $^{**}p < 0.01$, $^{***}p < 0.001$.

consistent with an activation of placental mTORC2 signaling in GDM. As both mTORC1 and mTORC2 are able to regulate placental amino acid transport (Rosario et al., 2013), our results suggest that increased placental nutrient transfer due to activation of mTORC1 and 2 may contribute to the increased fetal growth in GDM.

A pro-oxidant and pro-inflammatory environment has been found to be associated to pregnancy complications and intrauterine programming in GDM, pregestational diabetes, obesity and pre-eclampsia (Higa and Jawerbaum, 2013; Lappas et al., 2011; Myatt et al., 2014; Pantham et al., 2015). The placenta shows increases in oxidative stress and pro-inflammatory markers in different experimental models of pre-gestational diabetes and obesity (Jawerbaum and Gonzalez, 2006; Lappas et al., 2011; Pantham et al., 2015). The increased lipoperoxidation and nitric oxide production found in this work suggest increased oxidative and nitrative stress in the GDM placentas. Indeed, increased nitrated proteins, which suggests peroxynitrite-induced damage, was also observed in GDM placentas. Altogether, our results suggest that placental function

may be influenced by excessive oxygen and nitrogen species in the evaluated model of GDM induced by intrauterine programing. Putative links of the observed mTOR activation and the pro-inflammatory environment should be further studied. Indeed, TNFα has been recently shown to activate placental System A amino acid transport, and mTOR activation has been also related to inflammatory pathways in different pathological situations (Aye et al., 2015; Srivastava et al., 2015; Yin et al., 2015).

PPARs are nuclear receptors that regulate the expression of key genes that control metabolic, antioxidant and anti-inflammatory pathways (Wahli and Michalik, 2012). Fetal and placental growth, clearly related to the activation of mTOR pathways (Diaz et al., 2014; Roos et al., 2009) have been previously found to be regulated by PPARα activation (Martinez et al., 2011). Nevertheless, the interaction between mTOR and PPAR pathways during development is complex and thus further research is needed to fully understand their nature and implications.

Previous studies in human term placenta from GDM and type 2 diabetic pregnancies have shown reductions in PPARα and PPARγ gene and protein expression (Capobianco et al., 2013; Holdsworth-Carson et al., 2010; Jawerbaum et al., 2004). It was interesting to find that the same changes in PPARs expression are evident in the novel gestational diabetic model evaluated. As GDM in our study is the result of intrauterine programming induced by maternal diabetes, studies addressing putative epigenetic changes related to the observed alterations are warranted. In a recent work, it was found that obesity induced adverse intrauterine programming differentially if present before or during pregnancy (Sasson et al., 2015). Future work in this newly described experimental model of GDM will allow a better differentiation of the mechanism involved in intrauterine programming induced by pre-gestational and gestational diabetes.

In conclusion, exposure to maternal diabetes *in utero* programs GDM in the female offspring. This novel model of GDM is associated with placental mTOR activation, decreased expression of PPARs, increased pro-oxidant/pro-inflammatory environment in the placenta and fetal overgrowth. Because the metabolic and feto-placental phenotype in this model of GDM resembles the characteristics of human condition this model may prove useful to identify mechanisms involved and to test new intervention strategies in GDM.

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